Gene expression of *Aspergillus flavus* strains on a cheese model system to control aflatoxin production

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ABSTRACT

The expression of genes associated with aflatoxin biosynthesis by different *Aspergillus flavus* strains growing on a cheese model system has not been studied. To control aflatoxin biosynthesis, it would be useful to understand the changes in gene expression during cheesemaking and relate those changes to toxin production. The objective of this study was to evaluate the effects of pH, water activity, and temperature on the expression of 2 regulatory genes (*aflR* and *aflS*) and 1 structural gene (*aflP*) involved in aflatoxin biosynthesis, using 3 aflatoxigenic *A. flavus* strains growing on a cheese-based medium and reverse-transcription real-time PCR. The gene expression patterns were influenced by *A. flavus* strain and environmental conditions. The structural gene *aflP* and the regulatory genes *aflR* and *aflS* showed similar expression patterns in each *A. flavus* strain, but we also observed inter-strain differences. We observed the highest expression levels at 6 and 9 d of incubation by *A. flavus* strains CQ8 and CQ103, and saw a decrease in the days following. Strain CQ7 showed the lowest expression of these genes. We observed the highest expression levels of these genes at pH 5.5, water activity 0.95, and 20 to 25°C; strain CQ103 showed a different pattern for the *aflS* gene, with maximum expression at pH 6.0 on d 6 of incubation. For the 3 strains, we found a strong correlation between the relative expression of the *aflR* and *aflS* genes and the concentration of aflatoxins under conditions that simulated cheese ripening. Control strategies to avoid aflatoxin contamination during cheesemaking could use the detection of regulatory gene expression.

Key words: aflatoxin, *Aspergillus flavus*, ripened cheese, gene expression

INTRODUCTION

Aspergillus flavus is among the most important toxigenic mold species that produce aflatoxins (Bhatnagar et al., 2006). These mycotoxins are secondary metabolites that filamentous fungi can produce in foods under appropriate environmental conditions. Although *A. flavus* grows and produces mycotoxins at a wide range of temperatures and water activities (a_w), the ideal temperature for aflatoxin production is 28 to 30°C; production decreases dramatically below 25°C and approaching 37°C. Better fungal growth and toxin production are also detected at higher a_w levels: growth and spore germination rates slow at $a_w < 0.85$ (Schmidt-Heydt et al., 2009; Abdel-Hadi et al., 2012). The aflatoxins AFB₁ and AFB₂ are produced mainly by *Aspergillus flavus*, but AFB₁ is produced in higher amounts than the other aflatoxins, and it is considered to be the most potent natural hepatocarcinogen characterized to date (Wogan, 1992; Groopman and Kensler, 2005).

Although *A. flavus* normally contaminates maize, cereals, spices, and dried fruit (Arrus et al., 2005; Molyneux et al., 2007; Zinedine et al., 2007; Hernández-Hierro et al., 2009; Astoreca et al., 2012), its presence in cheese has been reported by several authors (Barrios et al., 1997; De Santi et al., 2010; Baranyi et al., 2015). As such, mycotoxins AFB₁, AFB₂, AFG₁, and AFG₂ have all been detected in cheeses (Taniwaki et al., 2001). Apart from these reports, however, few studies have documented the occurrence of aflatoxins as a result of mold contamination in cheese.

Recent studies have investigated how the activation rate and regulation of aflatoxin biosynthesis genes are affected by variations in temperature and a_w (Gallo et al., 2016; Bernáldez et al., 2017; Peromingo et al., 2017). The production of aflatoxins is always preceded by expression of the genes involved in the aflatoxin biosynthetic pathway. The key regulatory genes involved in aflatoxin production as a cluster are *aflR* and *aflS* (Abdel-Hadi et al., 2011). The *aflR* gene encodes a

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protein required for transcriptional activation of most of the structural genes in the aflatoxin biosynthetic pathway (Georgianna and Payne, 2009); the function of the *aflS* gene is still unclear.

Studies have produced conflicting results about the role of *aflS* and *aflR* transcription as determinants of aflatoxin biosynthesis (Schmidt-Heydt et al., 2009, 2010; Abdel-Hadi et al., 2011; Yu et al., 2011; Zhang et al., 2014). Gallo et al. (2016) found that *aflR* and *aflS* were highly expressed at maximum and minimum AFB₁ production.

Even so, the sterigmatocystin-o-methyltransferase gene (*aflP*), a structural gene demonstrated to be necessary for aflatoxin production (Rodríguez et al., 2012), is activated by the *aflR* gene product (Sweeney et al., 2000). Therefore, the expression of both regulatory and structural genes could be good indicators of aflatoxin production under different environmental conditions during the ripening of cheeses.

The expression of genes associated with aflatoxin biosynthesis by different *A. flavus* strains growing on a cheese model system has not been studied. Understanding changes in gene expression related to toxin production during cheesemaking might allow for the design of control strategies to prevent product contamination.

The aim of this study was to evaluate the effect of pH, a_w , and temperature on the expression of 2 regulatory genes (*aflR* and *aflS*) and 1 structural gene (*aflP*) involved in aflatoxin biosynthesis, using 3 aflatoxigenic *A. flavus* strains on a cheese-based medium and reverse-transcription real-time quantitative PCR.

MATERIALS AND METHODS

Fungal Strains

We used *A. flavus* CQ7, *A. flavus* CQ8, and *A. flavus* CQ103—3 strains that produce AFB₁ and AFB₂—isolated from a vegetable coagulant typical of the Mediterranean area (*Cynara cardunculus* L.) that has been used for cheesemaking (Casquete et al., 2017).

Inocula Preparation

The strains were initially grown on malt extract agar (Scharlab S.L., Barcelona, Spain) at 25°C for 7 d. A spore suspension of each strain was collected by adding 10 mL of sterile 0.05% (vol/vol) Tween 80 (Scharlab S.L.) to each mold plate and then rubbing the surface with a glass rod. The suspension formed was filtered through 2 layers of cheesecloth. The concentration of each spore suspension was quantified using a Neubauer chamber and a microscope (Olympus CX 400, Tokyo,

Japan), and adjusted to 10⁵ spores/mL with sterile water.

Experimental Settings

The cheese agar medium used in this study was prepared as described by Casquete et al. (2017). The pH of autoclaved basic medium was modified to 5.0, 5.5, and 6.0 by addition of 0.2 μ M filtered lactic acid. The medium was also modified by the addition of 286, 193, 101, or 0 mL of glycerol (Scharlab S.L.) per liter of medium to obtain to a_w of 0.90, 0.95, and 0.99, respectively. The a_w of the cheese agar medium was confirmed by using a Novasina LabMaster a_w meter (AG Lachen, Switzerland). Following the additions, the cheese agar was then immediately poured into Petri dishes and allowed to solidify at room temperature. Initial experiments were performed at a fixed temperature (25°C), 3 pH levels (5.0, 5.5, and 6.0), and a_w of 0.99, as commonly encountered in cheese and cheese products. Then we fixed the pH of the medium at 5.0, with a temperature of 25°C, to analyze the effects of different a_w (0.90, 0.95, and 0.99) on aflatoxin gene expression. Finally, we used media at conditions for maximum aflatoxin production (pH 5.0 and 0.95 a_w) to assess the effect of temperature (15, 20, 25, and 30°C). The cheese agar medium was incubated for up to 12 d after the end of the lag phase. Samples were taken every 3 d (3, 6, 9, and 12 d) after inoculation. For each experiment and condition, 2 biological replicates and 3 technical replicates were performed.

Gene Expression Studies

Harvested biomass was stored at –80°C until RNA was extracted. RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, St. Louis, MO). Samples of 250 to 500 mg of mycelium biomass were ground to powder in a pre-frozen mortar, and total RNA was purified as described in Peromingo et al. (2017). We used DNase I (Fermentas/Thermo Fisher Scientific, Leon-Rot, Germany) to remove genomic DNA contamination. A NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) was used to determine RNA concentration and purity.

Gene expression studies were performed by using reverse-transcription real-time quantitative PCR. First, cDNA was synthesized using about 500 ng of total RNA according to the PrimeScript RT Reagent kit protocol (Takara Bio Inc., Otsu, Shiga, Japan; <https://www.takarabio.com/products/real-time-pcr/reverse-transcription-prior-to-qpcr/primescript-rt-reagent-kit>), and then used this for quantitative PCR analysis.

Table 1. Nucleotide sequence of primers for real-time quantitative PCR assays

Gene	Primer	Nucleotide sequence (5'–3')	Product size (bp)	Reference
<i>aflP</i>	F-omt	GGCCGCCGCTTTGATCTAGG	123	Rodríguez et al., 2012
	R-omt	ACCACGACCGCCGCC		
<i>aflR</i>	AflRTaq1	TCGTCCTTATCGTTCTCAAGG	89	Medina et al., 2015
	AflRTaq2	ACTGTTGCTACAGCTGCCACT		
<i>aflS</i>	AflSqPCR1	TGGTGCGACCATATTTACA	94	Peromingo et al., 2017
	AflSqPCR2	GGTTGGGTACGAACTGTTT		
<i>β-TUB.JD</i>	F-TUBjd	TCTTCATGGTTGGCTTCGCT	98	Peromingo et al., 2017
	R-TUBjd	CTTGGGTCGAACATCTGCT		

Primer pairs F/R-omt, AflRTaq1/2, and AflSqPCR1/2 were used to amplify the structural gene (*aflP*) and the regulatory genes (*aflR* and *aflS*), respectively (Peromingo et al., 2017); the primer pair F/R-TUBjd amplified the β -tubulin (*β-TUB.JD*) gene, used as the endogenous control (Table 1).

The qPCR reactions were carried out using an Applied Biosystems 7300 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The assays were prepared in triplicate in MicroAmp optical 96-well reaction plates and sealed with optical adhesive covers (Applied Biosystems). The SYBR Green methodology was performed, using 6.25 μ L of SYBR Premix Ex Taq (Takara Bio Inc.), 0.05 μ L of 50 $\times$ ROX Reference Dye (Takara Bio Inc.), 10 μ M of each primer, and 2.5 μ L of each cDNA template in a final volume of 12.5 μ L. A negative control without DNA was also included in every run. An initial denaturation step of 10 min at 95°C was followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Melting curve analysis of the PCR products was performed by heating to 72 to 95°C and continuous measurement of the fluorescence to quantify the PCR product. We calculated the threshold cycle (Ct) value, corresponding to the PCR cycle number at which fluorescence was detected above threshold, using 7300 Fast system SDS software (Applied Biosystems).

To avoid errors caused by the addition of different amounts of total cDNA to each reaction, we quantified the expression of the *aflP*, *aflR*, and *aflS* genes with the housekeeping gene *β-TUB.JD* as an endogenous expressed control. We calculated the expression ratio using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). The experimental treatments used in this study have been shown not to influence the expression of the internal control gene (Peromingo et al., 2017). Evaluating relative gene expression by reverse-transcription real-time quantitative PCR allows for determination of the expression ratio of a target gene between the tested sample and its relative calibrator (“control” sample). The amplification efficiencies of the target and reference genes were practically equal (94.7% for the *aflP*

gene, 99.5% for the *aflR* gene, 99.5% for the *aflS* gene, and 86.6% for the *β-TUB.JD* gene). We used *A. flavus* samples incubated for 3 d as controls for temporal measurements of the expression of the *aflP*, *aflR*, and *aflS* genes.

Extraction and Quantification of Aflatoxins

We evaluated aflatoxin extraction and quantification by harvesting the biomass at 10 d after the end of the lag phase and storing it at -20°C until the aflatoxin extraction procedure. We extracted aflatoxins from the fungal mycelium using the quick, easy, cheap, effective, rugged, and safe (QuEChERS) procedure (Casquete et al., 2017), with modifications. For this purpose, 2 agar plugs of 4 mm in diameter were placed in an extraction tube containing 200 mg of MgSO_4 and 50 mg of NaOAc (Thermo Fisher Scientific). Then, 1 mL of HPLC-grade acetonitrile (Fisher Scientific) was added and the mixture was shaken vigorously for 2 min, followed by sonication for 20 min, and then centrifugation at $14,000 \times g$ at 25°C for 1 min. The supernatant was transferred to a clean tube containing 75 mg of MgSO_4 , 25 mg of primary secondary amine (Sigma-Aldrich), and 25 mg of C18 (Scharlab S.L.), and homogenized for 1 min. The mixture was then centrifuged ($14,000 \times g$, 25°C , 1 min) and 400 μ L of the supernatant was filtered through a filter (0.22 μ m pore size) into vials for quantification.

The AF analysis was carried out using an Agilent 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a diode array detector (G1315B; Agilent) set at 360 nm and using a C18 HPLC column (250 $\times$ 4.6 mm, 5 μ m particle size; Supelco, Bellefonte, PA). The mobile phase used for the separation contained HPLC-grade water (solvent A) and HPLC-grade acetonitrile (solvent B), run in a gradient mode set from 15% B at initiation to 100% B at 30 min. A 3-min period was necessary for column equilibration. All solvents used were purchased from Thermo Fisher Scientific (Runcorn, UK). The injection

volume was 25 μ L, and the flow rate was 1 mL/min for all experiments. Calibrations were carried out for each aflatoxin using standards purchased from Sigma-Aldrich (St. Louis, MO).

Statistical Analysis

We conducted statistical analysis of the parameters using one-way ANOVA. The means were separated by Tukey's honestly significant difference test ($P \leq 0.05$) in SPSS for Windows 21.0 (SPSS Inc., Chicago, IL). Levels of aflatoxins after 10 d of growth (AFB₁ and AFB₂) were correlated with the temporal relative expression of the *aflP*, *aflR*, and *aflS* genes in *A. flavus* CQ7, CQ8, and CQ103. The results from the principal component analysis (PCA) were interpreted graphically by drawing biplots, in which scatter plots of the score matrix (points) and the loading matrix (arrows) for the first 2 components were superimposed for a condensed representation of the correlation between the original variables and the distribution of the samples. The statistical significance was set at $P \leq 0.05$.

RESULTS AND DISCUSSION

Effect of pH, a_w , and Temperature on the Relative Expression of Genes Involved in Aflatoxin Biosynthesis by *A. flavus*

The involvement of abiotic factors in the activation of aflatoxin biosynthetic genes in different foods and synthetic food media has been previously studied, but not in a cheese model system. The effects of pH, a_w , and temperature on the relative temporal gene expression of *aflR*, *aflS*, and *aflP* genes by the 3 aflatoxin-producing *A. flavus* strains are presented in Figure 1, Figure 2, and Table 2.

In general, the expression patterns of the genes were influenced by environmental conditions. Because gene transcription occurs before toxin production, a temporal analysis of the expression of biosynthetic genes using real-time quantitative PCR, could help in taking early action to prevent aflatoxin production (Visentin et al., 2012; Rodríguez et al., 2014; Peromingo et al., 2017). Samples incubated for 3 d for each treatment were used

Figure 1. Effect of pH 5.0 (dotted line), 5.5 (solid line), and 6.0 (dashed line) on the temporal relative expression of *aflR*, *aflS*, and *aflP* genes by *Aspergillus flavus* grown on a cheese-based medium at a water activity level of 0.99 and a temperature of 25°C for 12 d after the end of the lag phase. The calibrator consisted of samples taken at d 3 of incubation and always had a value equal to 1. Error bars represent the statistical standard error.

Figure 2. Effect of temperature 30°C (dotted line), 25°C (solid line), 20°C (dashed line), and 15°C (dot-dash line) on the temporal relative expression of *aflR*, *aflS*, and *aflP* genes by *Aspergillus flavus* grown on a cheese-based medium at a water activity level of 0.95 and a pH of 5.0 for 12 d after the end of the lag phase. The calibrator consisted of samples taken at d 3 of incubation and always had a value equal to 1. Error bars represent the statistical standard error.

as calibrators; any expression before this time point was not considered in this study.

Figure 1 shows the effect of different pH levels (5.0, 5.5, and 6.0) at an a_w of 0.99 and a temperature of 25°C on the relative expression of aflatoxin biosynthesis genes

(*aflR*, *aflS*, and *aflP*) by *A. flavus* CQ8, CQ103, and CQ7 in a synthetic cheese medium during an incubation period of 12 d. We evaluated the relative expression of the target genes at different sampling times and compared them with controls (*A. flavus* CQ8, CQ103, and

Table 2. Effect of water activity level (0.99, 0.95, and 0.90) on the temporal relative expression of the *aflR*, *aflS*, and *aflP* genes by *Aspergillus flavus* grown on a cheese-based medium at pH 5 and 25°C for 12 d¹

Gene expression	Days	<i>A. flavus</i> CQ8			<i>A. flavus</i> CQ103			<i>A. flavus</i> CQ7		
		0.99	0.95	0.90	0.99	0.95	0.90	0.99	0.95	0.90
<i>aflR</i>	3	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	—
	6	0.455 ^a	1.012 ^a	1.132 ^a	0.152 ^a	26.446 ^b	2.090 ^a	0.167 ^a	0.139 ^a	—
	9	0.395 ^a	1.087 ^a	0.806 ^a	1.228 ^a	2.026 ^a	0.585 ^a	0.108 ^a	0.065 ^a	—
	12	0.292 ^a	0.578 ^a	0.890 ^a	0.702 ^a	0.001 ^a	0.001 ^a	0.261 ^a	0.167 ^a	—
<i>aflS</i>	3	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	—
	6	0.967 ^a	0.740 ^a	0.966 ^a	0.865 ^a	10.435 ^b	0.095 ^a	0.768 ^a	0.232 ^a	—
	9	1.235 ^a	0.869 ^a	0.975 ^a	1.227 ^a	0.781 ^a	1.228 ^a	0.827 ^a	0.087 ^a	—
	12	1.261 ^b	0.019 ^a	0.899 ^b	0.702 ^a	0.001 ^a	0.702 ^a	2.129 ^b	0.203 ^a	—
<i>aflP</i>	3	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	1.000 ^a	—
	6	0.573 ^a	0.657 ^a	3.264 ^b	1.254 ^a	0.781 ^a	0.993 ^a	0.847 ^a	0.220 ^a	—
	9	0.157 ^a	1.794 ^a	5.278 ^b	1.218 ^a	1.697 ^a	1.228 ^a	1.074 ^a	0.117 ^a	—
	12	0.197 ^a	0.014 ^a	2.567 ^b	0.702 ^a	0.001 ^a	0.702 ^a	2.651 ^b	0.296 ^a	—

^{a,b}Values with different superscript letters were significantly different ($P < 0.05$) between water activity levels within the same strain and day.

¹The calibrator consisted of samples taken at d 3 of incubation and always had a value equal to 1.

CQ7 samples after 3 d of incubation). The regulatory genes showed similar patterns of gene expression at the 3 pH conditions tested, with different transcriptional profiles between the 3 strains. For the CQ8 strain, we observed the highest expression values at 6 and 9 d of incubation at pH 5.5, with a decrease in expression levels over the following days. We observed a significant increase in expression of the regulatory genes at 6 d of incubation at pH 5.5 in strain CQ103. In contrast, CQ7 strain showed the lowest expression of these genes at all pH levels tested. This was not surprising, because the CQ7 strain has shown a relatively low capacity to produce aflatoxins in cheese media (Casquete et al., 2017). Gallo et al. (2016) and Rodríguez et al. (2014) have demonstrated that mycotoxin biosynthetic gene expression might be strain-dependent. In addition, the production of mycotoxins is usually higher at low pH: Keller et al. (1997) have reported higher aflatoxin production by *A. parasiticus* at pH 4.0 to 6.0 than at pH 7.0 and 8.0.

Figure 2 shows the effect of different temperatures (30, 25, 20, and 15°C) at 0.95 a_w and at pH 5.0 on the relative expression of the aflatoxin biosynthesis genes (*aflR*, *aflS*, and *aflP*) by the *A. flavus* strains in synthetic cheese medium during an incubation period of 12 d. In general, the regulatory genes showed similar patterns of gene expression at all temperatures tested, with different transcriptional profiles between the 3 strains. For the CQ8 strain, we observed the highest expression values on d 6 and 9 of incubation at 20°C, with a decrease in expression levels over the following days; the *aflS* gene showed the highest levels of expression for this strain. Strain CQ103 showed a significant increase in expression of regulatory genes *aflR* and *aflS*

at 6 d of incubation at 25°C, whereas *aflP* was the most expressed gene for this strain at 20°C and d 9 of incubation. In contrast, CQ7 showed an increase in expression of all 3 genes throughout the incubation period at 30°C; *aflR* was most highly expressed. Previous study on these strains reported optimal aflatoxin production at 25 to 30°C at 2 and 4 d of incubation (Casquete et al., 2017). However, in the current study, samples taken from d 3 were used as calibrators for the expression studies. Therefore, we did not assay activation of transcription before d 6 of incubation. Temporal analysis of the expression of biosynthetic genes to predict aflatoxin production would be feasible only if the window between gene activation and phenotypic expression was long enough. Clearly, the quantitative components of optimal aflatoxin production and relative transcript levels for the biosynthetic pathway genes chosen for this study were not temporally consistent. Peromingo et al. (2017) found the highest expression values for the *aflR* and *aflS* genes at 25 and 20°C and 0.95 a_w by *A. flavus* on a dry-cured ham-based medium.

Table 2 shows the effect of different a_w (0.99, 0.95, and 0.90) at 25°C and pH 5.0 on the relative expression of the aflatoxin biosynthesis genes tested. For strain CQ8, we observed the highest expression values of the *aflP* gene at 0.90 a_w , with a maximum value at 9 d of incubation. For strain CQ7, the *aflP* gene showed maximum expression at 0.99 a_w at the end of the incubation period (9 and 12 d). Strain CQ7 did not grow at 0.90 a_w , pH 5.0, and 25°C. In contrast, in the CQ103 strain, the *aflR* and *aflS* genes exhibited higher levels of expression at 0.95 a_w and d 6 of incubation. These results were consistent with those obtained by Casquete et al. (2017), in which the strains CQ7 and CQ103 were un-

Table 3. Effect of pH (5.0, 5.5, and 6.0) on aflatoxin production ($\mu\text{g}/\text{cm}^2$) by 3 *Aspergillus flavus* strains 10 d after the end of the lag phase¹

Factor	T (°C)	a_w	pH	Aflatoxin production ($\mu\text{g}/\text{cm}^2$)					
				<i>A. flavus</i> CQ7		<i>A. flavus</i> CQ8		<i>A. flavus</i> CQ103	
				AFB ₁	AFB ₂	AFB ₁	AFB ₂	AFB ₁	AFB ₂
pH assay	25	0.99	6.0	0.00 ^a	0.00 ^a	2.86 ^b	0.00 ^a	0.00 ^a	0.00 ^a
	25	0.99	5.5	0.00 ^a	0.00 ^a	2.95 ^b	0.00 ^a	1.55 ^b	0.00 ^a
	25	0.99	5.0	0.87 ^b	0.00 ^a	1.98 ^a	0.00 ^a	1.46 ^b	0.00 ^a
a_w assay	25 ²	0.99	5.0	0.87 ^a	0.00 ^a	1.98 ^a	0.00 ^a	1.46 ^b	0.00 ^a
	25	0.95	5.0	47.39 ^b	39.83 ^b	22.79 ^b	1.23 ^a	62.71 ^a	3.01
	25	0.90	5.0	—	—	0.99 ^a	—	—	—
T (°C) assay	30	0.95	5.0	23.38 ^c	3.03 ^b	126.41 ^c	3.29 ^c	145.12 ^d	4.65 ^c
	20	0.95	5.0	12.37 ^b	0.00 ^a	3.05 ^a	0.00 ^a	3.90 ^b	0.00 ^a
	25 ²	0.95	5.0	47.39 ^d	39.83 ^c	22.79 ^b	1.23 ^b	62.71 ^c	3.01 ^b
	15	0.95	5.0	0.00 ^a	0.00 ^a	0.38 ^a	0.00 ^a	0.38 ^a	0.00 ^a

^{a-d}Values with different superscript letters were significantly different ($P \leq 0.05$) between conditions within the same strain.

¹AFB₁ = aflatoxin B₁; AFB₂ = aflatoxin B₂; a_w = water activity; T = temperature.

²Data rows repeated for easier data comparison.

Figure 3. Principal component analysis of the relative expression of *aflR*, *aflS*, and *aflP* genes (diamonds) on a cheese-based medium by *Aspergillus flavus* strains CQ7, CQ8, and CQ103 and the levels of aflatoxin B₁ (B1) and aflatoxin B₂ (B2; circles) of the same samples determined after 10 d of incubation.

able to produce aflatoxins at 0.90 a_w , but CQ8 showed production. However, the 3 strains used in Casquete et al. (2017) showed different levels of expression at 0.95 a_w , where the maximum aflatoxin accumulation was evident. Other authors have found a strong induction of the *aflP* gene by *A. flavus* at 0.90 a_w (Peromingo et al., 2017).

In general, the levels of expression of the 3 genes *aflP*, *aflR*, and *aflS* were much higher in the *A. flavus* CQ8 and CQ103 strains than in the CQ7 strain. Gene expression was affected by pH, temperature, a_w , and interactions between these components. We observed the highest gene expression levels at pH 5.5, temperature 25°C and a_w 0.90 to 0.99; optimal a_w was strain-dependent.

Correlations Between Aflatoxin Production and Temporal Expression of Biosynthetic Genes

Figure 3 shows the results of the principal component analysis including the relative expression of *aflR*, *aflS*, and *aflP* at d 9 of incubation in combination with the amounts of AFB₁ and AFB₂ produced by the 3 strains of *A. flavus* on synthetic cheese medium after 10 d of incubation. The AFB₁ and AFB₂ content at d 10 of incubation are shown in Table 3. The *aflR* and *aflS* regulatory genes were located on the positive axis of main component 1, defined by the production of AFB₁, explaining 72.49% of the total variance; component 2 describes an additional 16.97% of the original variability of the data (Figure 3). The principal component analysis from *A. flavus* showed a clear relationship between the concentration of AFB₁ and the relative

temporal expression of the genes studied, but we found no association between the amounts of AFB₂ and relative gene expression. These results agreed with those reported by Bernáldez et al. (2017) and Peromingo et al. (2017), who found a strong correlation between the temporal relative expression of regulatory genes *aflR* and *aflS* and the production of AFB₁ by *A. flavus*.

CONCLUSIONS

The current study demonstrated that the relative expression of the biosynthetic genes *aflR* and *aflS* from 3 different strains of *A. flavus*, grown on model cheese medium, was related to phenotypic aflatoxin production. We observed different transcriptional profiles in the 3 aflatoxin-producing strains, related to their aflatoxin production. The relative expression of *aflR* and *aflS* from the aflatoxin biosynthetic pathway could be used to design and implement effective control strategies for avoiding aflatoxin production during the manufacture and storage of cheese.

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